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# Determination of Optical Density (OD) of Oligodeoxynucleotide from HPLC Peak Area

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**Abstract**: Oligodeoxynucleotides (ODNs) are typically purified and analysed with HPLC equipped with a UV-Vis detector. Quantities of ODNs are usually determined using a UV-Vis spectrometer separately after HPLC, and are reported as optical density at 260 nm ($OD_{260}$). Here, we describe a method for direct determination of $OD_{260}$ of ODNs using the area of the peaks in HPLC profiles.

**Keywords**: HPLC; OD; oligonucleotide; optical density; quantification

## Introduction

Oligodeoxynucleotides (ODNs) synthesized on an automated synthesizer are usually purified using reversed-phase (RP) or ion-exchange HPLC. Their purity is also usually determined using HPLC. For both preparative and analytical HPLC, the elution profile is mostly generated using a UV-Vis detector with the wavelength set to 260 nm. Quantities of ODNs are typically documented using optical density at 260 nm, which is abbreviated as $OD_{260}$. It is defined as the UV absorbance at 260 nm ($A_{260}$) of the ODN to be quantified dissolved in 1 mL of water with a light path of 1 centimetre. The value of $OD_{260}$ is usually determined separately after an ODN is purified by HPLC using an UV spectrometer or other UV based apparatus such as a NanoDrop. Because the peak area in an HPLC profile is a quantitative measure of the UV absorbance of the ODN eluted within the peak, we reasoned that a separate step for the determination of $OD_{260}$ using a UV spectrometer as we usually do is unnecessary, and the $OD_{260}$ value can be determined directly using the peak area in the HPLC profile. Here, we describe the establishment of a correlation graph between HPLC peak areas and $OD_{260}$ values and demonstrate its use for the determination of $OD_{260}$ using HPLC peak area without having to measure UV absorbance separately.

## Materials and Methods

Oligodeoxynucleotides (ODN) **1a** (20-mer, 5'- TCA TTG CTG CTT AGA CCG CT-3'), **1b** (21-mer, 5'-TTG CCA TGA TTG ACA ACC AAT-3') and **1c** (32-mer, 5'-TAG TTT TAT AAT TTC ATC AGC AGT GTT ACC GT-3') were either obtained from a commercial source or synthesized on a MerMade-6 DNA/RNA synthesizer at 1 (**1a**) or

0.2 (**1b-c**) μmol scale under standard synthesis, cleavage and deprotection conditions.[1] RP HPLC was carried out under typical conditions described elsewhere.[2] UV absorbance was obtained on a Horiba Scientific Duetta Fluorescence and Absorbance Spectrometer at 260 nm in a 1 mL quartz cuvette with a 1 cm light path. Water was used as the blank. UV absorbance was also obtained using a ThermoScientific NanoDrop 1000 Spectrophotometer at the same wavelength with water as the blank; the data are equivalent to those obtained using a spectrometer with a conventional 1 cm light path.

**Table 1.** HPLC peak areas and their corresponding $OD_{260}$ values.

| Entry | Volume | HPLC peak area | $OD_{260}$[1] | $OD_{260}$[2] |
|---|---|---|---|---|
| 1 | 0.5 μL | 97.7 | 0.03 | 0.04 |
| 2 | 2.5 μL | 422.6 | 0.16 | 0.16 |
| 3 | 4.0 μL | 585.7 | 0.24 | 0.24 |
| 4 | 6.0 μL | 829.6 | 0.31 | 0.32 |
| 5 | 7.5 μL | 1141.9 | 0.40 | 0.44 |
| 6 | 9.0 μL | 1372.4 | 0.50 | 0.53 |

[1] Measured with a UV-Vis spectrometer. The fractions from the corresponding HPLC peak were combined and evaporated to dryness. The ODN was dissolved in 1 mL of water and the UV absorption was measured using a 1 mL cuvette with 1 cm light path.
[2] Measured with a NanoDrop spectrometer using 2 μL of the same sample that was used for the measurement with the UV-Vis spectrometer.

For establishing the correlation graph of $OD_{260}$ vs HPLC peak area (Figure 1), ODN **1a**, which was synthesized at 1 μmol scale and purified with trityl-on RP HPLC, was dissolved in 1 mL water. Different volumes of the solution (see Table 1) were injected into HPLC. For each injection, the peak area of the ODN was recorded, and the fractions corresponding to the peak were collected, combined and concentrated to dryness in a centrifuge evaporator under vacuum. The ODN was dissolved in 1 mL water. UV absorbance at 260 nm, which is the $OD_{260}$ of the ODN in the corresponding HPLC peak, was obtained on a UV-Vis spectrometer using the solution in a 1 mL cuvette with a 1 cm light path, and on a NanoDrop Spectrometer using 2 μL of the solution (Table 1). The correlation graphs of the values of $OD_{260}$ vs the HPLC peak areas for the injections were generated and presented in Figure 1. The slopes of the lines obtained using $OD_{260}$ measured using a UV-Vis spectrometer and a NanoDrop spectrometer are 0.00036 and 0.00038, respectively.

For demonstrating the use of the graph for the determination of $OD_{260}$ from HPLC peak area, crude ODN **1b** was synthesized at 0.2 μmol scale on CPG, cleaved, deprotected, and purified with trityl-on RP HPLC. One twentieth of the ODN was injected into HPLC. The area of the ODN peak was found to be 1690.6, which corresponds to an $OD_{260}$ of 0.608 according to the line obtained using the UV-Vis spectrometer in Figure 1. Thus, the $OD_{260}$ for the 0.2 μmol ODN synthesis was 12.16. To validate the result, the $OD_{260}$ of the synthesis was also determined using standard method by measuring the absorbance at 260 nm on a UV-Vis spectrometer. The number obtained was 11.20. The use of the graph was further validated using ODN **1c** following the same procedure for **1b**. The $OD_{260}$ values calculated from the line obtained using the UV-Vis spectrometer and obtained a UV-Vis spectrometer were 16.92 and 15.40, respectively.

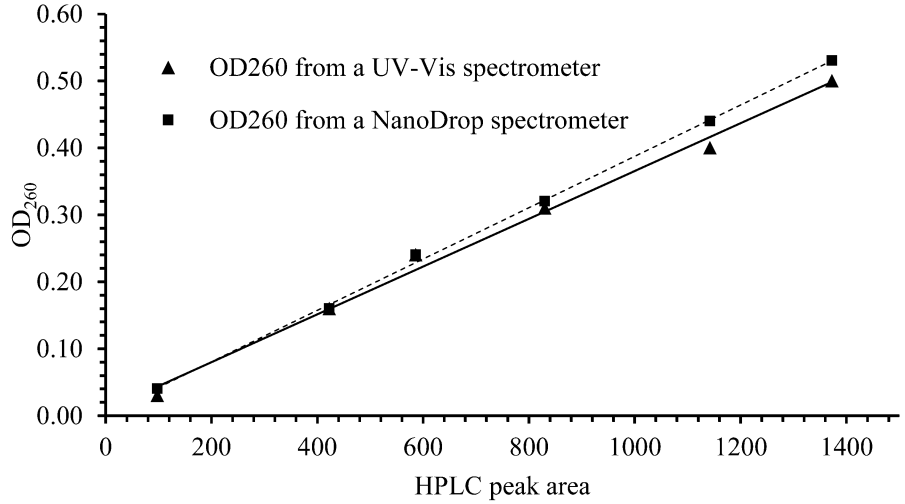


**Figure 1.** The correlation graph of the values of $OD_{260}$ and HPLC peak areas. The slopes of the lines obtained using $OD_{260}$ measured using a UV-Vis spectrometer and a NanoDrop spectrometer are 0.00036 and 0.00038, respectively.

## Results and Discussion

Oligonucleotides including oligodeoxynucleotides (ODN) are usually quantified by measuring UV absorbance at 260 nm ($OD_{260}$) in a separate step after HPLC purification or analysis. Once the value of $OD_{260}$ of an oligonucleotide is obtained, its mass in micro grams or micro moles can be easily calculated based on its sequence. Such calculations are usually carried out using free online tools by simply imputing the sequence and the $OD_{260}$ value. In this report, we describe a method to determine $OD_{260}$ directly from the area of HPLC peak instead of obtaining the value in a separate step.

To use HPLC peak area to determine $OD_{260}$, a correlation graph between $OD_{260}$ and HPLC peak area needs to be established first. ODN **1a** is used to demonstrate the process. A solution of **1a** from a 1 μmol synthesis was prepared (see Materials and Methods section). The concentration does not need to be known or accurate, but should be suitable for the generation of HPLC peaks with areas close to those in HPLC profiles a lab typically generates for ODN purification and analysis. For the case of **1a**, the ODN from the 1 μmol synthesis was dissolved in 1 mL water. Various volumes as indicated in Table 1 was injected into HPLC. For each injection, the ODN under the correct peak was collected and the area of the same peak was recorded (Table 1). The ODN was evaporated to dryness, and dissolved in 1 mL of water. The value of $OD_{260}$ was then obtained by measuring the absorbance using a UV spectrometer or a NanoDrop. Plotting the $OD_{260}$ numbers against peak areas gave the required correlation graph (Figure 1). As expected, the data fitted well with straight lines, and the two lines are very close. Their slopes are 0.00036 and 0.00038, respectively. The slight difference may come from the lower accuracy of NanoDrop measurements.

Once the correlation graph is obtained, the $OD_{260}$ for any ODN that has an HPLC profile, whether the profile is generated as a result of preparative or analytical HPLC, can be easily calculated. The ODN **1b**, which was synthesized at a 0.2 μmol scale, is used as

an example. One twentieth of the crude ODN was injected into HPLC. The area of the ODN peak was found to be 1690.6. Using the correlation line generated with the data from UV-Vis spectrometer in Figure 1, the area corresponds to an $OD_{260}$ of 0.608. Alternatively, when the concentration of the ODN solutions are not very high, and the correlation is a straight line, the slope of the correlation line can be determined, and $OD_{260}$ can be calculated from the slope. In present case, the slope is 0.00036, and the $OD_{260}$ is 0.608 (1690.6 × 0.00036). With the number for a portion of the sample, the $OD_{260}$ for the 0.2 μmol synthesis can be easily calculated, which is 12.16 (0.608 × 20). To validate the result, the $OD_{260}$ of the synthesis was measured in a standard way using a UV-Vis spectrometer, and the value was 11.20, which was close to the value calculated from the graph or the slope. The method was further validated using ODN **1c** using the same procedure for **1b**, and the numbers from the graph and from standard measurement were 16.92 and 15.40, respectively. In both examples, the $OD_{260}$ values from the standard measurements are slightly lower than those from the graph. This can be attributed to the inevitable loss of ODN during the collection of the samples from HPLC and subsequent material transfers before UV measurement.

Although the method for the determination of $OD_{260}$ is simple and convenient, it is recommended that the validity of the graph or slope is checked once in a while. This is especially important when HPLC conditions such as column, eluents, gradient, flow rate and temperature are changed. In addition, if the flow cell of the UV detector is replaced or cleaned, a new graph should be generated. Gratifyingly, in most labs, HPLC is typically performed under consistent conditions and UV detector flow cell can last for many years, there is no need to validate the graph frequently. Another thing we wish to point out is that when the area of the peak of an ODN is out of the areas used to generate the correlation graph, caution is needed to use the graph to calculate its $OD_{260}$ because the correlation may not be linear when the concentration of the ODN in the eluent is too high. A simple solution for this potential problem is to collect the fractions of the peak, determine the $OD_{260}$ in the standard way, and add that datum point to the graph (in this case, the graph may not be linear). Finally, although intuitive, it is important to point out that the usually long and thick waste eluent line connecting the UV detector flow cell to waste container needs to be removed when collecting fractions of eluent to generate the $OD_{260}$-HPLC peak area correlation graph. If this were not followed, the fractions collected may not be the fractions corresponding to the intended HPLC peak.

## Conclusion

In summary, a simple and convenient method for the determination of $OD_{260}$ of oligonucleotides using HPLC peak area is described. Although only quantification of ODN is described here, it is conceivable that the method can be equally applicable to RNA quantification. Because synthetic oligonucleotides typically have to go through the HPLC procedure for the purposes of purification or analysis, the direct $OD_{260}$ determination method can save time for researchers by bypassing the step of measuring UV absorbance using a spectrometer.

***Acknowledgement***

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